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14. ABSTRACT

Monoclonal antibodies are drugs that can specifically bind targets present on tumor cells. The highly aggressive triple-negative subtype of breast cancer does not have specific antibody drugs like Herceptin, and there is considerable need for targeted therapeutics and diagnostic biomarkers. We have developed a new technique allowing for discovery of new antibodies that disrupt a key process in cancer progression termed "epithelial to mesenchymal transition" or EMT. This process is important in several cancers, but is particularly associated with "triple-negative" breast cancer. We have applied our technology to identify unique antibodies that inhibit EMT and are now characterizing the antibodies to determine their targets on the cell. The newly discovered antibodies will then be engineered for utility as new highly specific drugs and diagnostics in preclinical experiments. This research could provide a new class of antibody therapeutic and diagnostic for triple-negative disease, identify new drug targets or pathways in cancer cells and make a major impact on breast cancer.

15. SUBJECT TERMS
Monoclonal Antibody, Epithelial to Mesenchymal Transition, Antibody Library

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1. **INTRODUCTION:**

Our proposal aims to "Revolutionize treatment regimens by replacing drugs that have life-threatening toxicities with safe, effective interventions", particularly for triple negative breast cancer which is often associated with epithelial-to-mesenchymal transition (EMT) features and has substantial need for new and effective therapeutics. Epithelial to mesenchymal transition is a process normally used during embryonic development where epithelial cells downregulate their tight junctions (and adhesive properties), degrade extracellular matrix, and effectively "invade" other tissues to migrate to new cellular locations. The EMT program involves transcription of multiple new genes and regulation of several proteins including intermediate filaments and cell-surface proteins. Not surprisingly, invasive cancer often co-opts this process and advanced cancer cells often show properties of EMT. Since EMT is an extremely important biological process in cancer progression, and since antibodies are proven therapeutic and diagnostic molecules, our goal is to use a novel EMT assay to select antibodies from newly developed lentiviral libraries that either inhibit or reverse EMT. Further, we will employ a new antibody structure derived from cows to identify novel antibodies against unique epitopes. We will determine the cellular target and effects of these antibodies, and evaluate their potential as therapeutic candidates *in vitro* and *in vivo*.

2. **KEYWORDS:**

Monoclonal Antibody

Lentiviral Library

Epithelial to Mesenchymal Transition

Ultralong CDR H3

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.*

What were the major goals of the project? The goals of the project encompass two specific aims:

Specific Aim #1: Generate unique antibodies that inhibit EMT and characterize their functional properties

Specific Aim #2: Develop anti-EMT antibodies for therapeutic use

The aims and specific tasks associated with them from the Statement of Work are described below in Table 1.

Table 1. Statement of Work goals, tasks, and progress

Goal	**Timeline (Months)**	**Progress**
Specific Aim #1: Generate unique antibodies that inhibit EMT and characterize their functional properties		
Major Task 1: Screen lentiviral antibody libraries		
Subtask 1: Construct new antibody libraries	1-3	(80% completed) We have constructed new vectors for bovine library insertion, have constructed part of this library and are currently completing it. Our experiments below used a fully human scFv-Fc library.
Subtask 2: Screen libraries for EMT inhibiting antibodies	4-10	(75% complete) We have screened for EMT inhibiting antibodies and have identified multiple candidates that appear to inhibit EMT. We are continuing to screen and confirm these results and attempting to identify the antigen to which they bind.
Subtask 3: Screen libraries for antibodies that induce EMT reversal	8-12	(20% complete) We have developed the assays for EMT reversal screening and are currently screening our libraries.
Milestone(s) Achieved: antibodies that inhibit or reverse EMT *in vitro*.	12	(75% complete). We appear to have antibodies that inhibit EMT, and are confirming these results.
Major Task 2: Identify targets of antibodies		
Subtask 1: Immunoprecipitation and MS of antigen	12-14	(55% complete) We are in the process of identifying the antigens for candidate antibodies.
Subtask 2: Confirm antigen binding	14-20	(25% complete)
Milestone(s) Achieved: Targets of anti-EMT antibodies are verified	20	(15% complete)
Specific Aim #2: Develop anti-EMT antibodies for therapeutic use		
Major Task 3: Analyze in vitro activity of antibodies		
Subtask 1: Evaluate cell growth inhibitory/killing,	20-24	(0% complete)

invasion, stem-cell self renewal and mammosphere formation properties of antibodies (standard cell lines, no identifiable information available to investigators)		
Subtask 2: Analyse mRNA pathways impacted by the antibodies by deep sequencing	20-24	(0% complete)
Milestone(s) Achieved: Cellular and molecular properties of inhibitory antibodies identified *in vitro*	24	(0% complete)
Major Task 4: Demonstrate anti-tumor activity *in vivo*		
Subtask 1: Evaluate efficacy in tumor xenograft models (3 groups of 8 mice)	24-36	(25% complete)
Milestone(s) Achieved: Antibodies identified that specifically inhibit tumor progression in vivo	36	(25% complete).
Local IRB/IACUC Approval	24	(100% complete)

What was accomplished under these goals?

We have summarized our progress in **Table 1** above, in association with the tasks in the Statement of Work. We were able to screen novel lentiviral antibody libraries to identify antibodies with EMT inhibitory properties, have constructed new libraries based on the cow antibody scaffold, and are now characterizing these antibodies.

Major Task 1

Subtask 1: Construct new antibody libraries

One of our goals is to employ the unusual diversity of cow antibodies into mammalian display and lentiviral selection systems. In this regard, we have constructed vectors with cow VH regions, including ultralong CDR H3s, human IgG1 constant regions, and the transmembrane region from bovine IgM. With a model antibody gene construct containing the germline VH-DH-JH recombination configuration, we previously showed that these unusual antibodies can effectively be displayed on the cell surface.

Additionally, we successfully prepared cDNA from lymphocytes derived from cow peripheral blood, spleen, and lymph nodes, amplified this cDNA by PCR with VH gene specific primers, and this "library" has been cloned into our display vector for surface display and functional selection of new cow antibodies. Furthermore, we are in the process of immunizng cows with the EMT cell line TES2b in order to produce selected antibodies that may bind EMT induced antigens.

Subtask 2: Screen libraries for EMT <u>inhibiting</u> antibodies

During the course of our studies we investigated a cellular model of EMT through two cell lines derived from a breast cancer patient, TES1 and TES2b. TES1 was derived from a primary tumor and has clear epithelial characteristics, and TES2b was derived from a pleural effusion at a later stage from the same patient, and has clear mesenchymal properties. These lines could be clinically relevant controls for the antibodies derived from the HMLE-Twist-ER EMT model. These lines were analyzed by (i) gene expression arrays, (ii) deep sequencing for cancer mutations. We identified two groups of genes that could be drug targets in these studies; the LRP8-ApoE axis, and aldo-keto-reductases.

Apolipoprotein E is a main apoprotein of the chylomicron that binds to the LDL receptor.

LDLR is expressed by hepatocytes as well as other peripheral cells and its ligand ApoE is essential for normal catabolism of triglyceride-rich lipoprotein constituents by mediating internalization of lipids into the cell. This mechanism provides the cell with high energy substrates for lipolysis, energy production, synthesis of cell specific lipids, e.g. those needed for milk production in breast epithelial cells, and other components required for cell survival, activity and proliferation. LRP8 is an endocytic receptor that mediates VLDL uptake and redistribution of lipids to cells for energy metabolism and cell signaling. ApoE overexpression has shown to be associated with a number of different cancers including ovarian and lung cancer. High expression levels of ApoE have been shown to promote cell proliferation and survival in OVCAR3 cells as well as non-cancer environments. Knock down of ApoE expression in OVCAR3 cells resulted in low cell proliferation and

increased apoptosis. Overexpression of ApoE in adipocytes showed increased protection from apoptosis, and increased cell proliferation and differentiation. Despite this data, there is very little literature regarding the role of ApoE and LRP8 in cancer.

Previously we selected antibodies via phage display to ApoE and LRP8 and have now identified their monoclonal sequences. We will be producing the IgG versions of these antibodies and analyzing their biochemical functions and activities in cell based assays.

We previously showed that a set of genes strongly overexpressed in EMT cells are from the aldo-keto reductase family (AKRs). Remarkably, 3 of the top 10 genes with induction in the mesenchymal TES2b cells

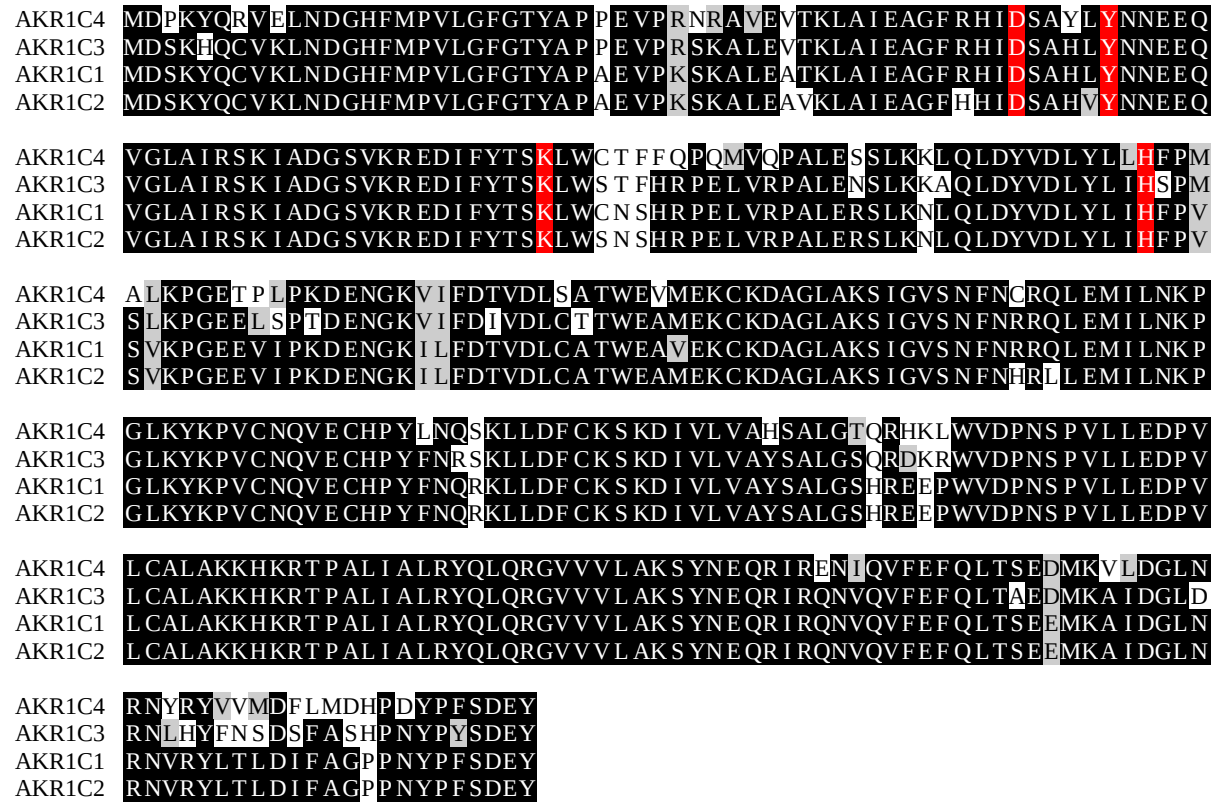

Figure 1. Amino acid sequence alignment of aldo-keto reductases highly overexpressed in TES2b cells with an EMT phenotype compared to TES1 cells with an epithelial phenotype. The conserved catalytic residues Asp50, Tyr55, Lys84 and His117 are colored red.

were from the AKR family. In an analysis of all 13 human AKRs, AKR1C3, AKR1C4, and AKR1C2 were all highly overexpressed in TES2b compared to TES1. AKR1C3 is known to be involved in testosterone and estradiol biosynthesis, as well as prostaglandin F synthesis and has previously been implicated in cancer progression, AKR1C2 is involved in androgen metabolism and is a potential drug target for prostate cancer. AKR1C4 is involved in bile acid synthesis but has not yet been implicated in tumor progression and EMT. We are following up on these interesting results, with particular emphasis on AKR1C4's role in EMT. AKR1C4's physiologic role is in the biosynthesis of bile acids; to our knowledge it has not been shown to play a role in cancer progression.

In the same screen for genes involved in EMT programming, we identified CXCL14 as being highly upregulated in TES2b. Our collaborator Dr. Brunie Felding has analyzed this factor in cell based and animal models, with initial indications that it plays a role in cancer invasion.

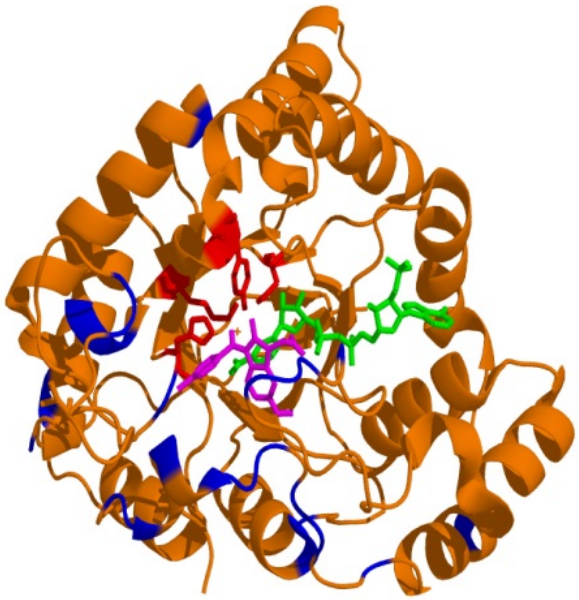

Figure 2. Variable residues could impact substrate usage in AKRs. The blue residues are variable positions between AKR1C1-4. These residues are near the active site and may play a role in substrate specificity. The structure is of AXR1C3 with the substrate indomethacin (pink) and NAD in green.

Subtask 3: Screen libraries for antibodies that induce EMT <u>reversal</u>

We have previously shown the development of assays for EMT reversal, with the completed construction of the new antibody libraries we will begin further screening.

Major Task 2

Subtask 1: Immunoprecipitation and MS of antigen

We have not yet confirmed antigen identify by MS, however, have identified LRP8 and ApoE antibodies by binding studies in subtask 2.

Subtask 2: Confirm antigen binding

We identified several genes upregulated in mesenchymal (TES2b) cells compared to epithelial (TES-1) cells, and embarked on an effort to confirm binding of antibody libraries to these targets. In particular ApoE and LRP8 are both highly up-regulated (111x and 3x, respectively) in mesenchymal cells. Library binding to these targets in phage format was evaluated after three rounds of selection on recombinant antigen. Two libraries were used: a synthetic germline library comprising ten VH and VL regions and highly diverse CDR H3s, and a "wellderly" library comprised of the antibody repertoires of hundreds of healthy elderly individuals without any chronic diseases (e.g. the "wellderly"). This latter library was constructed to take advantage of potentially highly robust immune systems of humans. Selection of these libraries revealed strong binding by the third round for ApoE for both the germline and wellderly libraries, and strong binding to LRP8 for the germline library (Figure XX).

Major Task 3

Subtask 1: Evaluate cell growth inhibitory/killing, invasion, stem-cell self renewal and mammosphere formation properties of antibodies

In the same screen for genes involved in EMT programming, we identified CXCL14 as being highly upregulated in TES2b. Our collaborator Dr. Brunie Felding has analyzed this factor in cell based and animal models, with initial indications that it plays a role in cancer invasion.

Subtask 2: Analyse mRNA pathways impacted by the antibodies by deep sequencing

Nothing to report

Major Task 4

Subtask 1: Evaluate efficacy in tumor xenograft models (3 groups of 8 mice)

In the same screen for genes involved in EMT programming, we identified CXCL14 as being highly upregulated in TES2b. Our collaborator Dr. Brunie Felding has analyzed this factor in cell based and animal models, with initial indications that it plays a role in cancer invasion.

What opportunities for training and professional development has the project provided?

Nothing to report.

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

We will express IgG of our anti LRP8 and ApoE antibodies, and evaluate their binding characteristics on purified antigen. We will also test their cancer inhibitory properties in cell based assays and potentially animal models.

We will analyze the role of AKR1C4 in cancer progression and drug resistance. We will express and purify AKR1C4, raise antibodies to it, and evaluate its ability to modify chemotherapy compounds (*e.g.* adriamycin). In transfection experiments we will evaluate whether AKR1C4 can induce drug resistance and/or enhance invasiveness of cancer cell lines.

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?

Currently there is nothing to report, however, we anticipate that the antibodies discovered will impact cancer treatment as well as provide unique reagents to study the EMT process. This will require verification in the studies of the next reporting period.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report. However, we anticipate that antibodies that show inhibitory activity in EMT animal models will be licenseable to a biotechnology or pharmaceutical company for drug development.

What was the impact on society beyond science and technology?

Nothing to report.

5. **CHANGES/PROBLEMS:** *The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change

The original six antibodies did not robustly recapitulate the ability to inhibit EMT. This could be due to (i) more than one sequence being selected from each clone, or (ii) more than one antibody contributing to the phenotype. We are are continuing to test whether these cells have more than one antibody.

Actual or anticipated problems or delays and actions or plans to resolve them

We have had a general delay in the progress of this project due to the unexpected attrition of a postdoctoral fellow to the biotech industry. We have hired a new postdoc for this position.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

   Nothing to report.

Publications, conference papers, and presentations

Nothing to report.

Journal publications.

Nothing to report.

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers, and presentations.

Nothing to report.

Website(s) or other Internet site(s)
Nothing to report.

Technologies or techniques
Nothing to report.

Inventions, patent applications, and/or licenses
Nothing to report.

Other Products
Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Vaughn V. Smider, M.D., Ph.D.
Project Role:	P.I. (unchanged)
Name:	Brunhilde Felding-Habermann, Ph.D.
Project Role:	Co-P.I. (unchanged)
Name:	Jeremy Haakenson, PHD
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	

Nearest person month worked:	1
Contribution to Project:	
Funding Support:	
Name:	Jayapriya Jayaraman
Project Role:	Research Technician
Nearest person month worked:	3
Funding Support:	
Contribution to Project:	
Name:	Wenyong Tong, Ph.D.
Project Role:	Postdoctoral fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	5
Contribution to Project:	Dr. Tong is expressing antibody sequences, and culturing cells.
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

The following grant was awarded and provides additional support for Dr. Smider:

NIH/USDA R01 HD088400 Smider (PI) 09/01/17-08/31/21
"Defining clinically relevant viral epitopes with cow antibodies"
The goal of this project is to identify and characterize bovine antibodies to unique conserved epitopes on the medically and agriculturally important viruses HIV and BVDV, respectively.

8. SPECIAL REPORTING REQUIREMENTS

Drs. Smider and Felding will be submitting separate versions of this report as co-PIs

What other organizations were involved as partners?

Nothing to report.

9. APPENDICES

Not applicable.